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NON-TECHNICAL SUMMARY

Epithelial cell plasticity in health and disease; relevance of somatic mutations in fate transitions.

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

Aging, Tissue regeneration, Early cancer formation, Genetic alterations, Stem cells

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main goal of this project is to understand how the cells that line the oesophagus (the gullet, which is the tube connecting your mouth to your stomach) and other similar tissues, like the skin, change and adapt when faced with challenges such as getting older, injury, genetic alterations or the start of cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The oesophagus is a vital organ, and diseases that affect it are unfortunately difficult to treat. Oesophageal cancer is one of the deadliest cancers worldwide, representing the 7th most common cause of cancer death (4th in males, 10th in females) with 9,525 new cases of oesophageal cancer cases in the UK every year (2019, 2021-2022). Traumatic injuries to the oesophagus also have poor prognosis, with a survival rate of less than fifty percent. This means that these medical conditions place a huge burden on patients, their families, and the public health system, as current treatments are often inefficient and unsuccessful. We need to learn more about this organ so we can find smarter ways to heal it and stop diseases before they take hold.

Our project tackles this problem by studying how the cells lining the oesophagus—and other similar cells, like those in your skin—handle stress. When a tissue is challenged by getting old, a bad injury, or the very start of cancer, its cells have to change their job or switch their identity. We call this cell switching/plasticity.

Using specialized techniques, in vivo and ex vivo models (tissues, organs or cells taken from an animal) that mimic the living tissue, we are focusing on a number of different challenges/perturbations, such as ageing, injury, genetic alterations and early cancer. We want to find the master controls inside the cells that trigger this switching. We are especially interested in how the accumulation of damage in our DNA (the molecule that carries the genetic instructions in our cells) as we age affects the response of cells to different challenges (proposed above). We need to figure out how cells, under normal conditions, protect themselves from these perturbations, and why those protective mechanisms sometimes fail leading to abnormal cell behaviour and cancer.

By answering these questions, we expect to discover new targets for medical treatments. Our ultimate goal is to improve tissue repair by minimizing scarring after injury, and to identify early detection markers for oesophageal cancer. This work will help us improve the quality of life for patients and contribute towards solving the major treatment challenges currently faced in the clinic.

What outputs do you think you will see at the end of this project?

Our project hopes to create two main outputs. That is, brand-new scientific knowledge, and strategies that doctors can use to fight medical conditions.

New Scientific Discoveries

Our biggest output will be scientific publications explaining how the cells in the oesophagus and skin handle serious challenges like aging, injury, and the very start of cancer. We aim to identify the 'molecular switches' (controls systems inside cells) that make them change when a tissue is stressed.

A crucial finding will be revealing the processes that normally prevent cells with 'DNA damage' (somatic mutations) from becoming cancerous as we age. We will figure out exactly how these barriers break down, which is essential information for cancer prevention. All the data we collect will be shared for free online. This open access helps other scientists around the world and reduces the need for them to repeat our animal experiments.

New Tools for the Clinic

These discoveries will lead directly to new medical solutions. By understanding the communication between healthy and damaged cells, we expect to find new targets for drugs. Our goals are to find early warning signs to diagnose cancer earlier or stop it in its tracks. And to improve healing of tissues, reducing scarring and contributing to regenerative medicine.

Who or what will benefit from these outputs, and how?

The outputs of this project will deliver substantial benefits across four key groups, including patients, the scientific community, healthcare providers, and industry.

Patients and Public Health- The most significant benefit is for patients suffering from highly aggressive conditions, such as oesophageal cancer and severe tissue injuries. Our ultimate goal is to translate our findings into new therapies, like drugs that can modulate how cells function to prevent/treat disease. By identifying new early detection markers for oesophageal cancer, we will directly improve patient survival rates. We also expect to develop better wound healing methods, leading to less scarring and improved regenerative medicine outcomes. This work reduces the overall social and economic burden these severe diseases place on public health systems.

Scientific Community and Research- In the short term, the scientific community will also benefit from this work. Our publications will provide novel, fundamental information about the molecular switches that control cell identity (plasticity) when tissues are stressed by aging, injury, genetic alterations or cancer. This knowledge will serve as a benchmark for research worldwide. We commit to making all our data publicly available, which not only speeds up discovery but also supports the 3Rs (Replacement, Reduction, Refinement) by allowing other labs to use our data instead of repeating animal experiments.

Healthcare and Industry- Healthcare providers will gain access to potential drug targets and new diagnostic methods. Industry will benefit by commercializing these outputs, using our discoveries to develop new drugs for both cancer and tissue repair and developing new experimental approaches.

How will you look to maximise the outputs of this work?

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We maximise the outputs of our work by doing the following:

● To increase the outputs of the work conducted within this programme, the management of individual projects is closely supervised by the project license holder. This avoids effort duplication and ensures that the resources are maximised and shared between members of the laboratory team, where feasible.

● By disseminating our observations from early stages, for example via conferences, we create new collaborations with experts in research fields beyond our own. They positively contribute to the progress of ongoing projects and ensure their completion to the highest standards. This has previously allowed us to establish collaborations with researchers interested in samples created as a side-product of our research. For example, samples are currently being shared with other groups examining the formation of cancer in other organs such as the tongue and the cheek, among others.

● Moreover, in our effort to understand the behaviour of cells, we conduct the analysis of molecular studies producing large amount of data (large-scale datasets). To maximize the outputs obtained from these complex datasets, we collaborate with experts in theoretical and mathematical modelling, who have the skills to develop theories explaining the results obtained in our experiments. Methods and datasets generated in these types of analyses are made publicly available as part of the relevant research articles enabling new collaborative projects.

● Our work will be published in free, public repositories online and in international scientific journals. In our publications, we do not only include positive data, but also experimental results that fail to support our hypotheses. This also prevents duplication by ensuring that other laboratories do not spend resources in ideas that have already been tested.

● We will share our work with the public via public engagement activities. This will allow people to learn about novel aspects of regenerative medicine and early cancer, and will provide the opportunity for a productive exchange of ideas between patients and scientists.

Species and numbers of animals expected to be used

- Mice: 20000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Traditionally, mice are used in our research field because they are mammalian pre-clinical models that allow us to study the biological processes underlying human diseases such as cancer. Mice share important anatomical, physiological and genetic similarities with humans, including tissue organisation, immune responses and mechanisms regulating wound healing and tumour development. This makes them more appropriate than non-mammalian models, such as zebrafish, for studying the behaviour of

stratified epithelial tissues such as the oesophagus and skin in a context that is highly relevant to human biology and disease.

Mice also allow relatively easy genetic manipulation, something that enables

i) injecting substances that enable us to track individual cells over time and follow their behaviour,

ii) manipulating cells genetically in order to understand the processes regulating their behaviour.

Our studies particularly focus on the oesophagus because of its structural simplicity. This facilitates the visualization and tracking of the cells we are interested in. Our results are then compared with other tissues, such as the skin, to identify whether our observations are relevant beyond the oesophagus.

This project will expand on our previous work by making use of all the methods that we have developed to study oesophageal/skin cell behaviour using animal models.

Mice will be studied at different stages of their lifetimes (from developmental, postnatal to adult and aged stages) because we are interested in understanding why we exhibit a different wound healing response and cancer incidence as we age.

In addition, the wide availability of established transgenic mouse lines and imaging approaches allows us to study cell behaviour in intact tissues over extended periods and across different stages of ageing. These tools are already well established within our research programme and allow us to build directly on previous work while minimising unnecessary animal use through the continued use of previous data and validated methodologies.

Typically, what will be done to an animal used in your project?

Some animals used in this project will be involved only in routine breeding and reproductive support procedures, including superovulation, embryo transfer, vasectomy and natural mating, to generate or maintain genetically altered lines required for the research. These are routine methods that are widely used in mouse breeding. Female mice given hormone treatment will usually receive two injections about 48 hours apart and may be mated. They will then be humanely killed at the appropriate time so that eggs or embryos can be collected after death. Female mice used to receive embryos will be mated with a sterile male and embryos will be transferred either without surgery (sometimes using brief anaesthesia) or during a short operation under general anaesthesia. These females will either be humanely killed if embryos are needed or allowed to give birth and care for their young. Male mice that have had a vasectomy will be cared for under veterinary supervision and humanely killed when they are no longer suitable for use. Animals used only for breeding are not expected to develop harmful effects, and small tissue samples may be taken to confirm their genetic type.

Most of our experiments will use genetically modified animals that allow us to follow the individual cells in which we are interested. This will facilitate the tracking of individual cells to study their behaviour in response to perturbations (including aging, wounding, genetic alterations or tumour formation). To address our research questions, animals will typically undergo one of three main types of experiments:

1-Aging: Animals will be administered various substances to label their cells, by injecting them into their abdomen or under their skin. This will help us track the behaviour of cells at different time points

throughout the lifetime of an animal. Samples will be collected after humanely killing mice at different ages from developmental stages and up to 24 months of age. The labelled cells will be analysed to investigate the impact of aging on cells.

2-Tumour formation

- Using chemicals: Animals will be treated with low doses of carcinogens (cancer-causing agents), typically via drinking water supplemented with sweeteners (such as Ribena) to alleviate any bitter taste.

- Tumour formation induced by genetic alterations: Alternatively, we will study how specific genetic changes contribute to the development and progression of cancer. To do this, we will use specially bred mice that carry genetic switches. These allow us to turn specific mutations on or off at chosen times. This approach helps us understand how cancer starts and behaves under different genetic conditions.

Animals in tumour protocols may also be injected with various substances to label their cells (as in 1). Animals will be allowed to grow old and tumours to form primarily in the skin and oesophagus. However, carcinogens may also induce sporadic tumours in other tissues, in which case these will serve to compare whether mechanisms are the same across tissues. Since we are particularly interested in early tumour formation, most animals are humanely killed before showing any signs of distress or suffering. Tumour samples will be collected from humanely killed mice using tissue dissection approaches at different times to capture the point at which tumours form. The labelled cells will be analysed to investigate how tumours emerge. Additionally, blood samples may be collected in order to identify markers of potential use to improve cancer detection in the clinic (from a superficial blood vessel or terminal sampling after humane killing the animal). A subset of animals will be allowed to develop advanced cancers in order to determine whether our observations may help predict or prevent disease progression, which is what will ultimately benefit patients.

To activate these changes or to label cells so we can track them, mice will receive substances (for which established protocols exist), typically by injection under the skin or into the abdomen. This will help us follow the activity and behaviour of cancer cells over time.

At various stages of the study, we will humanely kill mice to examine their tissues. Further analysis of their tissues allows us to understand how cancer changes and evolves over time and how age might influence the disease.

3-Wound healing: Animals will have a surgical procedure. Surgery will be completed under general anaesthesia so that animals remain in a state of sleep/unconsciousness throughout the procedure. During this procedure we will

- Wound the mouse skin typically via scalpel/surgical scissors incision or via surgical punch biopsy (up to two wounds of no more than 1 centimetre in size, separated by at least 1 centimetre). Wounds may or may not receive a graft (a piece of living tissue that is transplanted surgically). Grafted and non-grafted wounds will help us understand different regenerative/healing processes.

- Wound the mouse oesophagus using a well-established and characterised oesophageal endoscopic system, which consists of a miniature camera and specially designed fine instruments inserted via the mouth to allow direct visualisation and controlled injury of the oesophageal lining with small biopsy forceps, as previously published in 2012.

A small number of animals may be treated with various substances to label the cells and track their regenerative response. Subsequently, samples will be collected after humanely killing mice at different ages from birth and up to 15 months of age. These experiments will shed light on how wound healing changes as animals age.

For any of these typical experiments we may sporadically treat a small number of animals with drugs that alter cell behaviour to help us discover the processes that regulate how cells respond to perturbations. These drugs will be administered via injections to the abdomen and/or under the skin. These drugs may also be administered in the diet/orally (drop to the mouth), via drinking water and gavage.

What are the expected impacts and/or adverse effects for the animals during your project?

Please note the adverse effects presented here are based on our previous experience using the proposed techniques. We anticipate that our work will have a minimal impact on the health and welfare of the animals, and will not lead to long-lasting pain, suffering, or distress. The genetically modified mice used are not expected to show deviations from normal health.

1. Substances administered during the course of this project are not expected to cause any harms that affect the animal's day-to-day life. In certain instances, treated animals may experience transient episodes of weight loss up to 15%, from which they usually recover after 2-7 days.

2. Carcinogenesis. Cancer-causing agents (such as DEN, Diethylnitrosamine) or animals bearing specific mutation switches will be used in this project to study early tumorigenesis (the initial stages of tumour formation) and disease progression (towards more aggressive cancer stages). Based on our extensive experience using these genetic models, most mice do not show any signs of illness and do not tend to develop visible tumours after receiving the agent that switches specific mutations on or off. When tissue growths do occur, they are usually non-cancerous (benign) and do not affect the wellbeing of animals. In a small number of cases, some mice may develop more advanced tumours. These can occasionally lead to symptoms such as changes in movement or digestion, or fluctuations in weight. These cases are rare. Studying these more developed tumours is essential to understand how and why some benign growths eventually become cancerous. In addition, in some genetically altered mice given substances used to activate cancer-related mutation switches, swelling of areas such as the lips, mouth, eyelids or paws may occur several weeks after treatment.

The health and well-being of all animals, and the potential impact of tumours, will be routinely assessed through monitoring for adverse clinical signs. Individuals displaying the clinical signs will be humanely killed.

3. Wounding/grafting surgeries to the skin. These will be limited to wounds of up to 1 centimetre in diameter, with pain relief given and close monitoring of the animal, in order to ensure that the injury causes minimal distress to the animal. All surgeries are carried out under anaesthesia and animals

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receive pain relief medication that will remove any discomfort during the procedure and in the recovery period. Mice normally recover from surgeries within 10 - 15 minutes and show normal behaviour. Sporadically mice may display a small amount of swelling in the wounded/grafted area. This naturally disappears 7 days after the procedure. This swelling has not been observed to cause distress to the animals.

4. When neonates or juveniles (mice that have not been weaned) undergo a procedure (typical duration of 5-20 minutes), they are returned with their littermates to their mother immediately afterwards. The mother has always been observed to welcome her litter back.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice

- Mild: 90% (approx.)

- Moderate: 10% (approx.)

What will happen to animals used in this project?

- Killed
- Used in other projects
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal experimentation is required to obtain relevant information on cellular behaviour within the living organism. In order to identify treatments of relevance to improve wound healing and reduce tumour growth, we need to understand the way in which different cell types interact with each other. The oesophagus and skin are complex organs formed by different layers and tissue compartments that contain different types of cells. In response to an injury or genetic alterations leading to cancer, all these cells need to communicate in order to elicit a coordinated response that results in the healing of the wound or the formation of a tumour, respectively. Unfortunately, this complex communication network is something that so far cannot be fully explored merely by growing cells outside the animal. Additionally, most techniques that work with cells outside of living animals do not fully reflect their normal behaviour over long periods. Hence, animal work is necessary to disentangle how different

cells communicate and cooperate in health and disease; an aspect of critical importance to understand the basis of tissue regeneration and cancer.

Which non-animal alternatives did you consider for use in this project?

In my laboratory, we have developed methods to grow tissues outside the animal known as 3D organ cultures. These are small pieces of tissue or groups of cells grown in the laboratory in a way that closely mimics how they are arranged and behave inside the body. We can currently grow epithelial tissues/cells (e.g. from the skin/oesophagus), as well as underlying supporting tissues (called stromal cells). This approach represents an outstanding tool to replace the use of animals for short-term experiments studying wound healing and tumour formation. At the moment, all our projects make use of this technique to partially replace animal usage, wherever possible.

Why were they not suitable?

Tissues are formed by many different types of cells. We are interested in understanding how those different types of cells (such as epithelial, mesenchymal, and immune cells) interact with each other and contribute to aging, wound healing and tumour formation over long periods of time. Unfortunately, to date, by growing cells/tissues outside the animals, we are not able to fully mirror the complex cellular interactions and long-term changes in cell behaviour that take place in the animal. Hence, these 3D organ culture can only be used to partially replace animal work, for certain research questions within our programme of work, but not for all studies. Until then, experimentation with animals will still represent the gold standard to unveil these intricate processes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The predicted number of animals needed for this project has been based on the following:

1. Animal numbers have been worked out with the advice of expert statisticians. This ensures that our experiments are designed properly and that the results obtained have enough statistical power to draw meaningful biological conclusions.

2. Animal numbers have been calculated based on the animals used in my laboratory in similar work over the past 10 years. I have also accounted for an increase in the number of members that form my laboratory, something anticipated for the next five years.

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3. We have carefully considered the best way to make sure we keep the lowest number of different types of mice (strains) for breeding, while ensuring we have enough mice to use for experiments.

4. Test experiments, called pilot experiments (which use a smaller number of animals; typically 3) will be used to calculate the amount of a substance that we can safely inject into the animal. It is important to find the smallest amount of the substance that has an effect but that will not harm the animal in any way. This will then allow us to perform the intended experiment using only enough mice to be sure that the results we obtain are statistically significant.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken advice received from a local, qualified expert in statistics. This will make sure that each experiment produces statistically meaningful data. Where possible, all experiments are designed to get the most information using the least number of animals possible. We will also take into consideration the NC3Rs guidance and experimental design assistant tool (https://www.nc3rs.org.uk/experimental-design-assistant-eda; https://nc3rs.org.uk/3rs-advice-project-licence-applicants-reduction). Additionally, all experiments will be designed taking into account the PREPARE guidelines (a document that gives scientists advice on how to plan animal research and experiments).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We implement different measures to reduce the number of animals used. Among them:

- Efficient breeding techniques: We will breed our genetically modified mice in a way to minimize waste. Any types of genetically modified mice (strains) that are not being used for scientific studies will be frozen as embryos for future use.

- Experimental design: Critical experiments are designed in collaboration with experts on statistical physics. With their assistance, we determine the number of experimental animals required to answer each of our scientific questions.

-Mouse strain management: animals are bred only to fulfil our experimental needs. However, any unavoidable excess of animals is used for in vitro experiments (culturing cells outside the animal), validation or pilot studies (as long as they are compatible). The latter represents a critical consideration in our laboratory, and allows us to ensure that mouse waste is minimised as much as possible. While this approach requires a significant amount of coordination and team effort, it is essential to reduce the number of animals we breed or purchase. We have joined the internal Colony Management Group to gain insight from the best practice guidance for optimising the use of animals and avoiding wastage.

- Animal and tissue sharing: I have a network of collaborators within the UK who work in my or a similar research field. We exchange particular types of mice (strains) between us to reduce animal imports, the need for additional breeding and its associated excess. Similarly, to increase the output of our work, we share tissues with members of other research groups and collaborators who examine different areas of the body (tissues), such as the tongue and the cheek. Additionally, we are registered in the animal tissue sharing list of our institution.

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- Growth of tissues outside the animal (3D tissue cultures): Using this method, one single oesophagus can produce sufficient cells to perform 3 experiments instead of one. Thereby, this system effectively reduces the number of animals used by two thirds. However, as indicated above, studying interactions between different types of cells remains a limitation when using this technique.

- Mathematical modelling: By working with our collaborators, we use our data to develop mathematical models that explain how cells behave and interact with each other. The created hypotheses are then tested in the laboratory. This makes our science more targeted, which allows us to significantly reduce the number of animals needed to understand how cells work.

- Advanced imaging and molecular methods: Dissected samples are analysed using state-of-the-art whole-tissue imaging and molecular techniques that require minimal tissue material to obtain meaningful results.

- Optimization of skin transplantation technique (grafting): We have implemented diverse adjustments in skin transplantation experiments, which increase the quality and statistical value of our data while substantially reducing the number of animals required.

-Randomisation and blinding are implemented wherever possible to increase the robustness of our observations. They help reduce bias by ensuring that researchers do not know which groups are which and that results are assessed objectively.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically modified (GM) mice to carry out our plan of work. Most of our GM mice bear genetic alterations that allow us to label the cells allowing us to track their behaviour in the tissue. Hence, the genetic alterations we propose are not expected to lead to any adverse effects.

- We **administer substances** to mice to label and track cells over time. These methods are based on previous studies and our long-term expertise using them. We use the lowest dose needed to observe an effect. Hence, we expect our treatments to cause minimal harm to animals.

- We also use drugs to understand how cells work and explore the earliest stages of **tumour formation** (carcinogens). Given our interest in early cancer, these treatments use low doses of these drugs. Our experiments have been optimized for over a decade, which allow most mice to live without complications for over a year. Carcinogen administration is typically performed non-invasively via drinking water.

- For **injury and tissue transplantation** (grafting) experiments. These methods have been significantly refined in my laboratory over the last 10 years. For oesophagus wounding, we developed an endoscopic technique which is the most refined approach available. It represents a significant refinement compared to other techniques used in the literature to wound the oesophagus, including the use of dental tools or treatment with acids. For skin wounding, we use instruments such as “punch” biopsies, scalpels, surgical scissors or biopsy forceps, which are specially designed to collect tissue specimens in the clinic. These instruments allow us to perform small skin wounds that favour rapid healing and diminish animal discomfort. Over the years, we have also optimised the size of the grafts, reducing it when compared to standard practice. We currently perform grafts of a maximum of one centimetre in diameter with optimal results. The most significant improvement of our refined method resides in the bandaging used to protect the wounded area. For this, we use transparent medical dressing (e.g. Tegaderm), which constitutes a very thin transpirable barrier that protects the wound and keeps it aseptic, while allowing animals to move freely. This significantly reduces the stress experienced by the mice while recovering from surgery. When compared to classical bandaging, transparent medical dressing has the added advantage of allowing constant monitoring of the wounded area after surgery. Animals typically remove the dressing by themselves on day 4-5 after surgery; by then, the exposed wound/graft allows for closer monitoring and mice are completely freed from any dressing.

Why can’t you use animals that are less sentient?

A critical aspect of our work focuses on investigating how different cell types interact and impact on each other's behaviour. For these studies to be clinically relevant, they need to be performed in mammalian animals, closer to humans. Results in less sentient such as fish, worms or fruit flies, would be of very limited medical relevance.

In particular, in our studies, we investigate how different cell types function under normal circumstances, and how they change their behaviour in response to injury and early cancer. Given the differences observed in wound repair and cancer incidence as we age, it is important to investigate cell behaviour over long periods of time and at different stages throughout the life of animals (from birth to aging). Unfortunately, these long-term experiments require living organisms, limiting the use of other less sentient animals such as anaesthetised mice or tissues grown outside the animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All methods in this project will use techniques that reduce animal stress and make sure the animal does not suffer as detailed below:

Surgery procedures:

- Surgery will be carried out in a clean manner (using aseptic technique). We will make sure to meet the level set out in the Home Office Minimum Standards for Aseptic Surgery and the LASA Guidance on Preparing for and Undertaking Aseptic Surgery (2017).

- We will ensure that animals suffer as little as possible during surgical procedures by giving the animals medication to manage their pain under the advice of the Named Veterinarian Surgeon.

Animals undergoing surgery will receive extra comfort measures after the procedure, including soft bedding, temporary heated pads, and mash to support recovery. They will be moved back with their cage mates immediately after recovery.

- Animals will be monitored before and after surgery to ensure that any deviation from normal health is picked up. Observations will be monitored on a chart. Animals will receive additional pain relief medication as needed, and advice from the Named Veterinarian Surgeon will be sought if animals show any deviation from normal behaviour.

- Wounding of the skin is performed using surgical scissors, or single-use medical tools called "punch biopsy tools" that have a sharp circular edge and allow to gently remove a small round piece of skin. These specialised medical tools allow to produce clean skin biopsies, which favours rapid healing and diminishes animal discomfort. The size of the grafts has been optimised to increase grafting success. The refinement of this technique also allows us to reduce the number of animals used.

- Skin wounds will be on body sites excluding face and paws to minimise discomfort.

- We have refined the skin wounding technique to use minimal bandaging/dressing, in order to avoid any restriction of animal movement while protecting the implant until grafted.

- We will use endoscopic biopsy to wound the oesophagus, currently the most refined approach available. We developed this technique together with close collaborators. It represents a significant refinement compared to other techniques used in the literature to wound the oesophagus, including the use of dental tools or treatment with acids.

General:

- We will make sure to use the best care methods to improve the quality of life for the animals. Mice will be placed in cages and will be provided with nesting and bedding material to stimulate mouse activity and provide them with shelter if needed.

- Mice are social animals and prefer to live in groups. For this reason, single housing will be avoided whenever possible. Keeping mice in groups helps reduce stress and supports their normal behaviour and wellbeing.

- To keep the mice comfortable and in a stable environment, the temperature and humidity in the animal room will be carefully controlled. Noise levels will be kept as low as possible, and a regular light/dark cycle will be maintained.

- Non-aversive handling i.e. using a tunnel or cupping in the hands to pick up mice rather than by the tail which is known to cause stress and anxiety.

- Side effects from procedures will be monitored by regularly weighing animals, daily health checks, and the use of scoring sheets, as required. These measures will help minimise animal suffering. If an animal begins to look unhealthy, it will be monitored more frequently and provided with pain relief or other minor interventions where appropriate. Animals showing signs of suffering that cannot be resolved within 48 hours using no more than minor interventions, or clinical signs that impair normal behaviour, will be humanely killed. This is called the Humane Endpoint. We do not expect animals undergoing procedures under this licence to experience severe suffering.

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- Some substances have to be given to animals by injecting them in specific places. We have therefore asked to be allowed to use different administration routes (e.g. intraperitoneal [injection into the abdomen], oral [via the mouth] and subcutaneous [injection under the skin]) in this project. We will always use the least harmful route possible to give an animal a substance. This is to make sure we cause the smallest amount of discomfort or pain to the animal. When possible, refined administration methods will be used to avoid any animal discomfort, including micropipette-guided voluntary oral administration or administration of substances in diet as an alternative to injections. In cases where drugs are given via drinking water, sweetened water will be provided to alleviate drug taste.

- All animals that are brought into the animal facility will be allowed at least 7 days to get used to their surroundings. This process is called acclimatisation. We will also allow them to get used to the animal technicians prior to use. This will reduce the amount of stress the animal experiences and will improve their well-being.

- To improve the quality of life of our animals, we always house them in groups, unless strictly necessary due to experimental reasons. Animals have enrichment in their cages (such as wood sticks, bedding and nesting material) for extra comfort and enhanced mental and physical health.

- Ageing mice will receive increased cage enrichment, and in the case of male mice, housing in groups of four will be prioritised as experience from other researchers has shown that lower cage occupancy leads to an increase in males fighting.

- Higher rates of unexpected deaths have recently been detected in animals carrying a specific gene modification (in the Gt(ROSA)26SorR2PI3K allele). We are actively investigating the potential underlying cause by analysing the animals' tissues post-mortem, and have already found that this problem can be reduced by breeding them with wild type animals to refresh the line, strategy that we are already implementing for animal colonies carrying this allele. Moreover, most mice will be kept with only one copy of the gene modification (called "heterozygous"). Mice with two copies of the gene modification (called "homozygous") may have a higher risk of harmful effect, and are therefore only produced when necessary for the research, and in small numbers. We will also monitor these mice more closely so that any signs of sickness can be picked up as early as possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the guiding principles on good practice for Animal Welfare. Our experiments are planned following the "Planning Research and Experimental Procedures on Animals: Recommendations for Excellence" (PREPARE) and "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) guidelines. We attain to the LASA (Laboratory Animal Science Association) guidelines, as well as the NC3Rs published strategy for improving animal welfare (see publications details below).

1. [redacted] (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

2. - LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. [redacted] http://www.lasa.co.uk/publications/

3. [redacted] and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Animal 51(1S): 5-57. doi: 10.1177/0023677217744587

4. [redacted] PREPARE: guidelines for planning animal research and testing. Lab Animal 52(2): 135-141. doi: 10.1177/0023677217724823.

For surgical procedures we follow the Laboratory Animal Science Association (LASA) Guidance on Preparing for and Undertaking Aseptic Surgery (2017) and the Home Office Minimum Standards of Aseptic Surgery.

For the breeding of genetically altered mice, we will follow the guidelines provided by the Home Office and the NC3Rs Resources on 'Genetically altered mice' detailed in:

1. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773553/GAA_Framework_Oct_18.pdf

2. https://www.nc3rs.org.uk/GAmice

Additionally, for cancer studies, we will refer to:

[redacted] ommittee of the National Cancer Research Institute. Guidelines for the welfare and use of animals in cancer research (2010). Br J Cancer 102(11): 1555-1577. doi: 10.1038/sj.bjc.6605642.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

-At our institution, we count on the outstanding support of our Biofacility Service. They keep us informed (through their central team) about new developments on 3Rs and offer us expert advice on how to implement them in our ongoing studies (via our very experienced team of animal technicians, Named Animal Care & Welfare Officer (NACWO), Named Veterinarian Surgeon (NVS) and Named Information Officer (NIO) all of them with significant expertise in animal care & welfare of animals used in scientific experimentations.

-We follow the website of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs, available at https://nc3rs.org.uk/resource-hubs). This allows us to stay up-to-date on the most relevant information.

-We also use our Institutional 3Rs search tool. This contains an up-to-date database with information on the best ways to reduce or replace animals in our experiments. It also contains advice on how to refine methods in order to reduce animal stress.

Additional guidance and information on the most appropriate and refined techniques for our studies may be obtained from external sources, including:

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- Laboratory Animal Science Association (LASA)
- Institute of Animal Technology (IAT)
- Norecopa (https://norecopa.no/databases-guidelines)
- Relevant literature

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